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**METHODS AND TOOLS FOR IDENTIFYING COMPOUNDS WHICH
MODULATE ATHEROSCLEROSIS BY IMPACTING LDL-
PROTEOGLYCAN BINDING**

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5 The invention was funded in part by National Institutes of Health program project grant HL41633. The U.S. Government may have certain rights to this invention.

10 TECHNICAL FIELD

15 This invention relates to the disease atherosclerosis, methods of modulating the formation of atherosclerotic lesions, and methods of identifying compounds which modulate atherosclerotic lesion formation. Specifically the invention relates to the reduction of atherosclerosis through the modulation of LDL-proteoglycan binding at Site B (amino acids 3359-3369) of the apo-B100 protein in LDL.

BACKGROUND ART

20 High levels of LDL are a major risk factor for coronary disease and are the source for most of the cholesterol that accumulates in the arterial wall (Ross, R. 1995. *Annu. Rev. Physiol.* 57:791-804). Subendothelial retention of LDL has been suggested to be a key pathogenic process in atherosclerosis, and several lines of circumstantial evidence suggest that intramural retention of atherogenic lipoproteins involves the extracellular matrix, chiefly proteoglycans (Hurt-Camejo, E. et al. 1997. *Arterioscler Thromb Vasc Biol.* 17:1011-1017; Williams, K. J., and I. Tabas. 1995. *Arterioscler. Thromb. Vasc. Biol.* 15:551-561; and Radhakrishnamurthy, B. et al. 25 1990. *Eur. Heart J.* 11 Suppl E: 148-157).

The significance of the possible LDL proteoglycan interaction has been highlighted in two recent review articles (Hurt-Camejo, E. et al. 1997. *Arterioscler*

proteoglycans increases during atherosclerosis and correlates with an increased accumulation of aortic cholesterol also supports the potential importance of the interaction between LDL and proteoglycans (Hoff, H. F., and W. D. Wagner. 1986. *Atherosclerosis*. 61:231-236; Merrilees, M. et al. 1990. *Arteriosclerosis*. 81:245-254).

Proteoglycans contain long carbohydrate side-chains of glycosaminoglycans, which are covalently attached to a core protein by a glycosidic linkage. The glycosaminoglycans consist of repeating disaccharide units, all bearing negatively charged groups, usually sulfate or carbohydrate groups. In vitro, LDL bind with high affinity to many proteoglycans found in the artery wall, including dermatan sulfate proteoglycans (e.g., biglycan) and chondroitin sulfate proteoglycans (e.g., versican), which are produced by smooth muscle cells in response to PDGF or TGF β (Schonherr, E. et al. 1991. *J. Biol. Chem.* 266:17640-17647; and Schönherr, E. et al. 1993. *Arterioscler. Thromb.* 13:1026-1036). The interaction between LDL and proteoglycans have been hypothesized to involve clusters of basic amino acids in apo-B100, the protein moiety of LDL, that interact with the negatively charged glycosaminoglycan proteoglycans (Mahley, R. et al.. 1979. *Biochem. Biophys. Acta.* 575:81-91; Camejo, G. et al. 1988. *Arteriosclerosis*. 8:368-377; Weisgraber, K., and S. Rall, Jr. 1987. *J. Biol. Chem.* 262:11097-11103; and Hirose, N. et al. 1987. *Biochemistry*. 26:5505-5512) or by bridging molecules such as apo-E or lipoprotein lipase (Williams, K. J., and I. Tabas. 1995. *Arterioscler. Thromb. Vasc. Biol.* 15:551-561).

Isolation of large fragments of apo-B100 from different regions characterized by concentrations of positive clusters indicated that up to eight specific regions in apo-B100 bind proteoglycans (Camejo, G. et al. 1988. *Arteriosclerosis*. 8:368-377; Weisgraber, K., and S. Rall, Jr. 1987. *J. Biol. Chem.* 262:11097-11103; and Hirose, N. et al. 1987. *Biochemistry*. 26:5505-5512). Weisgraber, K., and S. Rall, Jr. 1987. *J. Biol. Chem.* 262:11097-11103 identified two fragments, residues 3134-3209 and

3356–3489, that bind to heparin with the highest affinity. Recently Camejo and coworkers confirmed this finding and proposed that residues 3147–3157 and 3359–3367 may act cooperatively in the association with proteoglycans (Hurt-Camejo, E. et al. 1997. *Arterioscler Thromb Vasc Biol.* 17:1011-1017; and Olsson, U. et al. 1997. *Arterioscler. Throm. Vasc. Biol.* 17:149-155). However, because these studies were carried out with delipidated apo-B fragments in the presence of urea or with short synthetic apo-B peptides, it is not clear which of the binding sites are functionally expressed on the surface of LDL particles. Some or many of these postulated glycosaminoglycan-binding sites may not be functional when apo-B is associated with LDL. For example, apo-E has two heparin-binding sites, but only one binds to heparin when apo-E is completed with phospholipid (Weisgraber, K. et al. 1986. *J. Biol. Chem* 261:2068-2076). This heparin-binding site coincides with the LDL receptor-binding site of apo-E.

Although eight potential glycosaminoglycan-binding sites have been identified in apo-B100 (Camejo, G. et al. 1988. *Arteriosclerosis*. 8:368-377; Weisgraber, K., and S. Rall, Jr. 1987. *J. Biol. Chem.* 262:11097-11103; and Hirose, N. et al. 1987. *Biochemistry*. 26:5505-5512), it was not known which of them participate in the physiological binding of LDL to proteoglycans. Previously, we have demonstrated, in conjunction with others, that Site B (residues 3359-3369) is the LDL receptor-binding site, and in the study which generated the present invention we found that it is also the primary binding site to proteoglycans.

Modification of LDL potentially exposes the other proteoglycan-binding sites. Paananen and Kovanen (Paananen, K., and P. T. Kovanen. 1994. *J. Biol. Chem.* 269:2023-2031) noted that proteolysis of apo-B100 strengthened the binding of LDL to proteoglycans, suggesting the exposure of buried heparin binding sites. Likewise, when LDL are fused by sphingomyelinase treatment, the modified lipoproteins bind more avidly to proteoglycans. The finding that multiple heparin molecules bind to LDL (Cardin, A. et al. 1987. *Biochemistry*. 26:5513-5518) may also be explained by

a cooperative effect of heparin binding to one site that triggers a conformational change in apo-B100 that enables other sites to participate in the interaction. Thus, the initial interaction with proteoglycans may induce structural alterations of the LDL that expose heparin/proteoglycan-binding sites that may contribute to the intramural retention of LDL after the initial interaction with the primary binding site.

The interaction between LDL and the LDL receptor plays a major role in determining plasma cholesterol levels in humans and other mammalian species (Goldstein, J. et al., 1985. *Annu. Rev. Cell Biol.* 1:1–39). Apo-B100 is the major protein component of LDL and is responsible for the binding of these lipoproteins to the LDL receptor (Innerarity, T. et al. 1990. *J. Lipid Res.* 31:1337–1349). The relevance of this catabolic pathway is best illustrated by the genetic disorders familial hypercholesterolemia (FH) and familial defective apo-B100 (FDB), in which high levels of LDL accumulate in the circulation because mutations in the LDL receptor (FH) or in the ligand (FDB) disrupt the binding of LDL to its receptor (Innerarity, T. et al. 1990. *J. Lipid Res.* 31:1337–1349). Many different mutations of the LDL receptor cause FH (Hobbs, H. et al. 1992. *Hum. Mutat.* 1:445–466), but FDB is associated with a single site mutation, the substitution of glutamine (Innerarity, T. et al. 1987. *Proc. Natl. Acad. Sci. USA.* 84:6919–6923) or, in a few cases, tryptophan (Gaffney, D. et al. 1995. *Arterioscler. Thromb. Vasc. Biol.* 15:1025–1029) for the normally occurring arginine at residue 3500 of apo-B100. With the exception of an arginine-3531 to cysteine mutation (Pullinger, C. et al. 1995. *J. Clin. Invest.* 95:1225–1234), which is associated with a minor decrease in LDL receptor binding, extensive searches have not found any other mutations of apo-B100 that cause defective receptor binding of LDL (Pullinger, C. et al. 1995. *J. Clin. Invest.* 95:1225–1234). The FDB mutation occurs at an estimated frequency of 1/500 in the normal population and is therefore one of the most common known single-gene defects causing an inherited abnormality (Innerarity, T. et al. 1990. *J. Lipid Res.* 31:1337–1349).

Much attention has focused on understanding the molecular interaction between apo-B100 and the LDL receptor. The structural and functional domains of the LDL receptor have been defined in detail (Hobbs, H. et al. 1992. *Hum. Mutat.* 1:445-466), but much less is understood about the receptor-binding domain of apo-B100, because of its large size and insolubility in aqueous buffer. Furthermore, both the lipid composition and the conformation of apo-B100 appear to be crucial to its function as an effective ligand for the LDL receptor, since apo-B100 binds to the LDL receptor only after the conversion of large VLDL to smaller LDL (Goldstein, J. et al.. 1985. *Annu. Rev. Cell Biol.* 1:1-39).

Selective chemical modification of the apo-B100 of LDL demonstrated that the basic amino acids arginine and lysine were important in the interaction of LDL with its receptor (Mahley, R. et al.. 1977. *J. Biol. Chem.* 252:7279-7287; and Weisgraber, K. et al. 1978. *J. Biol. Chem.* 253:9053-9062). Once apo-B100 was sequenced, several regions enriched in arginine and lysine residues became candidates for receptor binding, including Site A (residues 3147-3157) and Site B (residues 3359-3367) (Knott, T. et al. 1985. *Science.* 230:37-43).

While it had been hypothesized that LDL-proteoglycan binding was possibly important to the formation of atherosclerotic lesions through the retention of lipoproteins in the subendothelium, this hypothesis has not been empirically demonstrated in the art. Moreover, there have been six obstacles which have prevented other researchers from demonstrating the mechanism by which atherogenesis occurs and using this information to combat atherosclerosis. First, there have been eight potential sites identified in the apo-B100 protein, any one or several of which could have been responsible for proteoglycans trapping LDL in the subendothelium. Second, it has been unknown which potential sites in the apo-B100 are exposed to the surface of the LDL particles and which are buried within the lipid core. Third, there has been evidence that some of the potential proteoglycan binding sites on apo-B100 may work cooperatively, creating the possibility that blocking

proteoglycan binding at any single site might not have proven both necessary and sufficient to eliminate LDL retention in the subendothelium. Fourth, the modification of LDL has been shown in some cases to expose new proteoglycan binding sites to the surface. Fifth, any disruption to LDL proteoglycan binding had the potential to disrupt LDL receptor binding, which would serve to disrupt the natural clearance of LDL from blood, raise serum cholesterol levels, and potentially result in a condition similar to familial hypercholesterolemia. Sixth, it has not been possible to use site-directed mutagenesis and express the entire mutated apo-B100 proteins as LDL in order to define the proteoglycan-binding sites on LDL.

SUMMARY OF THE INVENTION

We have discovered that the amino acids of Site B in the apo-B100 protein are responsible for conferring proteoglycan binding activity on LDL. Recombinant LDL in which lysine₃₃₆₃ in apo-B100 was changed to glutamic acid has severely defective proteoglycan binding activity but normal LDL receptor-binding activity. Thus, the proteoglycan-binding and the receptor-binding activities in LDL can be separated by the introduction of a single point mutations into the apo-B 100 protein, indicating that pharmaceutical strategies for disrupting LDL-proteoglycan binding need not inhibit LDL receptor binding.

Moreover, we have demonstrated for the first time *in vivo* that LDL-proteoglycan binding is necessary to the formation of atherosclerotic lesions and the onset of atherosclerosis. Transgenic mice expressing the mutant RK3359-3369SA apo-B100 LDL, which is defective for proteoglycan binding, was found to have strikingly less atherosclerosis than mice expressing the wild-type recombinant LDL, when both were fed a high cholesterol diet. These results demonstrate that disruption of LDL-proteoglycan binding at Site B in the apo-B100 protein is a credible target for pharmaceutical intervention for the reduction and elimination of atherosclerosis.

polynucleotide, LDL, apo-B100 protein, or protein fragment of the invention. Normally, a polynucleotide encoding an apo-B100 protein or protein fragment of the invention is transduced into a cell. The cell may be transduced *ex vivo*, then transferred into the animal or mammal, or the cell may be transduced *in situ*.

5 The present invention further encompasses methods of screening for and identifying inhibitors of LDL-proteoglycan binding, including drug screening assays based on simple LDL-proteoglycan binding, high through-put drug screening assays based on LDL-proteoglycan binding, two step LDL/proteoglycan and LDL/LDL-receptor binding assays, and in transgenic animals which express recombinant LDL.

10 The present invention encompasses methods for identifying inhibitors of LDL-proteoglycan binding, comprising the steps of:

 (a) incubating a mixture comprising (i) proteoglycan, (ii) LDL, and (iii) a candidate compound, under conditions wherein LDL binds to proteoglycan to form an LDL-proteoglycan complex in the absence of said candidate compound;

15 (b) determining any difference between the amount of LDL-proteoglycan complex present in:

 (i) the mixture prepared in step (a), and

 (ii) a control mixture comprising said proteoglycan and said LDL in the absence of said candidate compound; and optionally

20 (c) correlating any difference determined in step (b) with said candidate compound's ability to affect LDL-proteoglycan binding.

 The present invention also encompasses identifying compounds which affect LDL-proteoglycan binding, which do not substantially affect LDL receptor binding, which further comprising the steps of:

25 (d) incubating a mixture comprising (i) LDL receptor, (ii) LDL, and (iii) a candidate compound that affects LDL-proteoglycan binding identified in step (c), under conditions wherein LDL binds to LDL receptor to form an LDL-LDL receptor complex in the absence of said inhibitor of LDL-proteoglycan binding;

(e) determining any difference between the amount of LDL-LDL receptor complex present in:

(i) the mixture prepared in step (d), and

5 (ii) a control mixture comprising said LDL receptor and said LDL in the absence of said inhibitor of LDL-proteoglycan binding; and optionally

(f) correlating any difference determined in step (e) with the LDL-LDL receptor binding activity of said candidate compound that affects LDL-proteoglycan binding.

10 In accordance with the instant invention, either the LDL or the proteoglycan of step (a) may be adhered to a solid support. Additionally, where the LDL is adhered to a solid support, the proteoglycan may be labeled, or where the proteoglycan is adhered to a solid support, the LDL may be labeled.

15 The invention further encompasses methods for identifying compounds which modulate atherosclerosis and/ LDL-proteoglycan binding *in vivo*, comprising the steps of:

(a) administering a candidate compound to a transgenic non-human animal which expresses a human apo-B gene, under conditions wherein measurable atherosclerotic lesions form in the arteries of said animal in the absence of said candidate compound;

20 (b) determining any difference between the extent of atherosclerosis present in:

(i) the animal of step (a), and

(ii) a control transgenic non-human animal in the absence of said candidate compound; and optionally

25 (c) correlating any difference determined in step (b) with the said candidate compound's ability to modulate atherosclerosis *in vivo*.

The present invention further encompasses the compounds identified by the screening methods of the invention, including the compounds which affect, modulate,

stimulate or inhibit of LDL-proteoglycan binding identified by the methods for identifying compounds that affect LDL-proteoglycan binding, as well as the compounds that affect, modulate, stimulate, or inhibit LDL-proteoglycan binding, which do not substantially affect LDL receptor binding identified by the methods for identifying inhibitors of LDL-proteoglycan binding, which do not eliminate LDL receptor binding, and the compounds which modulate, stimulate, or inhibit atherosclerosis *in vivo* identified by the methods for identifying compounds which modulate atherosclerosis *in vivo*. In addition the invention encompasses methods of inhibiting atherosclerosis in a human comprising administering to the human an agent that inhibits LDL-proteoglycan binding, or any of the other compounds identified by the methods of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a half tone reproduction of a Coomassie staining and western analysis of recombinant LDL. Recombinant LDL ($d = 1.02-1.05$ g/ml) from four lines of human apo-B transgenic mice were isolated by sequential ultracentrifugation and subjected to immunoaffinity chromatography to remove endogenous apo-B and apo-E. Five micrograms of apo-B 100 from human plasma LDL (lane 1) or recombinant LDL: control LDL (lane 2), R3500Q LDL (lane 3), RK3359-3369SA LDL (lane 4), and K3363E LDL (lane 5) were analyzed by SDS-PAGE with 3-15% gels (Figure 1A). One microgram each of unpurified LDL (lane 1) and control LDL (lane 2), R3500Q LDL (lane 3), RK3359-3369SA LDL (lane 4), and K3363E LDL (lane 5) were analyzed by western blots with monoclonal antibody 1 D 1 against human apo-B (Figure 1B) and polyclonal antibodies against mouse apo-B (Figure 1C) or mouse apo-E (Figure 1D).

Figure 2 is a graph demonstrating a competitive binding assay of recombinant LDL. The abilities of recombinant control LDL (closed triangle), R3500Q LDL (closed square), RK3359-3369SA LDL (open diamond), and K3363E LDL (open

circle) to compete with ^{125}I -labeled human plasma LDL (2 $\mu\text{g}/\text{ml}$) for binding to LDL receptors on normal human fibroblasts were determined. The recombinant lipoproteins were isolated from 15 mice, and endogenous apo-E and apo-B were removed. Competitor LDL were added at the indicated concentrations to normal human fibroblasts, and the amount of ^{125}I -LDL bound to the fibroblasts was measured after a 3-h incubation. The results represent the average of data from three independent experiments performed with freshly isolated LDL for each experiment human plasma LDL (closed circle) was included as a control.

Figure 3 is a graph of a gel-shift analysis of mouse-derived recombinant LDL with (^{35}S)biglycan and (^{35}S)versican. The abilities of recombinant control LDL (closed triangle), R3500Q LDL (closed square), RK3359-3369SA LDL (open diamond), and K3363E LDL (open circle) to interact with (^{35}S)biglycan (Figure 3A) and (^{35}S)versican (Figure 3B) were determined. The recombinant lipoproteins were isolated from 15 mice, and endogenous apo-E and apo-B were removed by immunoaffinity chromatography. The results represent the average data from three independent experiments performed with freshly isolated LDL for each experiment. Human plasma LDL (closed circle) was included as a control.

Figure 4 is a graph of a gel-shift analysis of mouse-derived recombinant LDL with (^{35}S)versican and (^{35}S)biglycan after selective modification. The abilities of recombinant control LDL (closed circle) and RK3359-3369SA LDL (closed diamond), cyclohexanedione-modified control LDL (closed triangle, point down) and RK3359-3369SA LDL (closed triangle, point up), and acetylated control LDL (open circle) and RK3359-3369SA LDL (closed square) to interact with (^{35}S)versican (Figure 4A) or (^{35}S)biglycan (Figure 4B) were determined. The recombinant lipoproteins were isolated from 15 mice, and endogenous apo-E and apo-B were removed by immunoaffinity chromatography. The isolated recombinant LDL were treated with (Figure 4A) acetic anhydride or (Figure 4B) cyclohexanedione to selectively modify all arginines or lysines, respectively, in apo-B 100.

Figure 5 is a graph demonstrating the correlation between the percentage of total aortic surface area covered by lesions and the plasma cholesterol levels of transgenic mice expressing either normal human recombinant LDL (open circle) or defective- proteoglycan-binding LDL (closed circle) after the mice had been fed a high-fat, high-cholesterol diet for 17 weeks.

Figure 6 is a half-tone reproduction of photographs of Sudan IV-stained aorta from a mouse expressing human wild-type recombinant LDL (top), proteoglycan-binding-defective LDL (center), and endogenous LDL (bottom). The wild-type recombinant LDL mouse and the RK3359-3369SA LDL mouse had plasma cholesterol levels of 678 and 616 mg/dl, respectively.

DISCLOSURE OF THE INVENTION

The practice of the present invention encompasses conventional techniques of chemistry, immunology, molecular biology, biochemistry, protein chemistry, and recombinant DNA technology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Oligonucleotide Synthesis (M. Gait ed. 1984); Nucleic Acid Hybridization (B. Hames & S. Higgins, eds., 1984); Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989); PCR Technology (H.A. Erlich ed., Stockton Press); R. Scope, Protein Purification Principles and Practice (Springer-Verlag); and the series Methods in Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.).

Definitions:

The terms "LDL" or "low density lipoprotein" refers to a particle with a diameter of approximately 22 nm and a mass of about three million Dalton found in plasma. LDL is comprised of a highly hydrophobic core of approximately 1500 cholesteryl ester molecules surrounded by a shell of phospholipids, unesterified cholesterol, and a single apo-B100 protein. LDL is often differentiated and separated from other plasma lipoproteins by its density of 1.019 to 1.063 g/ml through

ultracentrifugation as described in Example 4. As used herein the term "LDL" embraces lipoprotein particles comprising a mutant apo-B100 protein, as well as lipids which do not naturally occur in LDL and labels, all of which may change the physical properties listed above. In all cases an LDL particle contains only one apolipoprotein, a apo-B100 protein or fragment thereof, and contains a lipid core which is predominantly cholesteryl ester.

The "apo-B100 protein" resides in the outer shell of very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and low density lipoproteins (LDL). The complete sequence and identification of structural domains of human apo-B100 protein is found in Knott, T. et al 1986. *Nature* 323:734-738.

Apo-B100 is the component of LDL which binds specifically to the "LDL receptor" on the plasma membrane of non-hepatic cells. The LDL receptors are localized in specialized regions called coated pits, where the "LDL/LDL receptor complex" is internalized through endocytosis, delivering cholesterol to the cell. As used herein the "LDL receptor" and any resulting "LDL/LDL receptor complex" need not contain any portion of the native LDL receptor which is not needed to achieve LDL-binding. Thus, only a sufficient portion of the 292 amino-terminal amino acid LDL-binding domain of the native LDL receptor and any other domains which are necessary to confer binding to LDL need be present in an "LDL receptor."

As used herein, the term "purified apo-B100 protein" refers to an apo-B100 protein isolated from a lipoprotein, including wild-type apo-B100, mutant apo-B100 and protein fragments thereof, which is essentially free, i.e., contains less than about 50%, preferably less than about 30%, and more preferably less than about 10%, even more preferably less than about 5%, and still more preferably less than about 1% of the lipids with which an apo-B100 protein is normally associated in a lipoprotein.

As used herein the term "Site B" refers to amino acids from about 3359 to about 3369 of the human apo-B100 protein

recombinant LDL which comprises the mutant protein and transgenic mice or other non-human animals which express the K3363E recombinant LDL.

As used herein the term "RK3359-3369SA" refers to a human apo-B100 protein in which the basic amino acids in Site B (residues 3359-3369) were converted to neutral amino acids with all of the arginine residues being converted to serines and the lysine residues being converted to alanines. The term is also used to refer to genes and plasmids which encode the RK3359-3369SA mutant apo-B100 protein, as well as recombinant LDL which comprises the mutant protein and transgenic mice or other non-human animals which express the RK3359-3369SA recombinant LDL.

As used herein the term "proteoglycan receptor⁺" is used to refer mutant apo-B100 proteins, fragments thereof as well as LDL comprising these polypeptides and transgenic non-human animal strains which express these proteins. A proteoglycan receptor⁺ apo-B100 protein when present in an LDL particle reduces proteoglycan binding of that LDL particle by at least 50%, preferably by at least 60%, more preferably by at least 70%, still more preferably by at least 80%, even more preferably by at least 90%, most preferably by 95% or greater. Proteoglycan binding may be assayed by any method known in the art. See, for example, the method described in Example 8. In addition a proteoglycan receptor⁺ apo-B100 protein when present in an LDL particle confers LDL receptor binding activity to that LDL particle of at least 60% of wild-type levels, preferably at least 70%, more preferably at least 80%, still more preferably 90%, most preferably at essentially 100% of wild-type LDL receptor binding activity.

The amino acid sequence of the wild-type human apo-B100 protein from amino acid 3358 to 3367 is as follows:

Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-Lys₃₃₆₃-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇.

The proteoglycan receptor⁺ mutant apo-B100 proteins of the invention involve substitutions or deletions at the following four amino acid positions: Lys₃₃₆₃, Arg₃₃₆₂, Arg₃₃₆₄, and Arg₃₃₅₉. A proteoglycan receptor⁺ mutant of the invention can

be constructed by the substitution or deletion of any single one of these amino acids, any combination or them or all four of the amino acids in positions 3363, 3362, 3364, and 3359. Preferably two or fewer of these amino acids are substituted or deleted, more preferably only a single amino acid is substituted or deleted. When only a
5 single amino acid is chosen to be substituted or deleted, preferably the amino acid which is substituted or deleted is one of positions 3363, 3362, and 3364, more preferably position 3363. While any amino acid can be used in a substitution, preferably the new amino acid is chosen from the group consisting of Gly, Ala, Val, Leu, Ile, Phe, Tyr, Trp, Cys, Met, Asn, Gln, Asp, and Glu, more preferably the new
10 amino acid is either Asp or Glu.

In addition to deletions and substitution, a proteoglycan receptor⁺ apo-B100 proteins can be formed by additions to the amino acid sequence. Additions are usually only a single amino acid, and can be made to one or more of the following locations: between 3358 and 3359, between 3359 and 3360, between 3361 and 3362,
15 between 3362 and 3363, between 3363 and 3364, and between 3364 and 3365. Preferably additional amino acids are added to two or fewer of these sites, more preferably an addition is made to only one of these sites. When only a single position is chosen for an addition preferably that site is either between 3362 and 3363, or between 3363 and 3364. While any amino acid may be added for these additions,
20 preferably amino acids for addition are chosen from the following list Gly, Ala, Val, Leu, Ile, Phe, Tyr, Trp, Cys, Met, Asn, Gln, Asp, and Glu, more preferably the new amino acid is Ala, Ser, Thr, Gln, Asp or Glu, even more preferably the new amino acid is Asp or Glu. It should be noted that combinations of the additions, deletions and substitutions described can be employed to construct a proteoglycan receptor⁺
25 apo-B100 protein. These changes to the native protein may be achieved by any method known in the art, including chemical synthesis or modification. However, expression of recombinant apo-B100 gene made by site-directed mutagenesis as demonstrated, for example, in Examples 1-3 is preferred.

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B3

The following are the amino acid sequences from position 3358 to position 3367 for a list of preferred proteoglycan receptor⁺ apo-B100 protein mutants:

5 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-**Glu**₃₃₆₃-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-**Asp**₃₃₆₃-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 5 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-**Ala**₃₃₆₃-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-**Thr**₃₃₆₃-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-**Ser**₃₃₆₃-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-**Gln**₃₃₆₃-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-**Glu**₃₃₆₂-Lys₃₃₆₃-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 10 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-**Asp**₃₃₆₂-Lys₃₃₆₃-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-Lys₃₃₆₃-**Glu**₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-Lys₃₃₆₃-**Asp**₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 Thr₃₃₅₈-**Glu**₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-Lys₃₃₆₃-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 Thr₃₃₅₈-**Asp**₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-Lys₃₃₆₃-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 15 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-----Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-----Lys₃₃₆₃-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-Lys₃₃₆₃-----Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-**Glu**-Lys₃₃₆₃-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-Lys₃₃₆₃-**Glu**-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 20 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-**Asp**-Lys₃₃₆₃-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇
 Thr₃₃₅₈-Arg₃₃₅₉-Leu₃₃₆₀-Thr₃₃₆₁-Arg₃₃₆₂-Lys₃₃₆₃-**Asp**-Arg₃₃₆₄-Gly₃₃₆₅-Leu₃₃₆₆-Lys₃₃₆₇,
 wherein the repeated dashed lines represent deletions.

The term "proteoglycan" refers to a class of compounds with a high relative molecular mass which comprise carbohydrate and protein, and are found in animal structural tissues, e.g. the ground substance of cartilage and bone. The ground substance and gel fluids of these tissues owe their viscosity and elasticity to the presence of proteoglycans. Each proteoglycan contains 40 to 80 mucopolysaccharide chains (glucosaminoglycans) usually bound to the protein via o-glycosidic linkages to

certain purposes higher resolution can be provided by using HPLC or other means well known in the art.

As used herein, the term "antibody" means an immunoglobulin molecule or a fragment of an immunoglobulin molecule having the ability to specifically bind to a particular antigen. Antibodies are well known to those of ordinary skill in the science of immunology. As used herein, the term "antibody" means not only intact antibody molecules but also fragments of antibody molecules retaining antigen binding ability. Such fragments are also well known in the art and are regularly employed both *in vitro* and *in vivo*. In particular, as used herein, the term "antibody" means not only intact immunoglobulin molecules of any isotype (IgA, IgG, IgE, IgD, IgM) but also the well-known active (i.e., antigen-binding) fragments F(ab')₂, Fab, Fv, scFv, Fd, V_H and V_L. For antibody fragments, see, for example "Immunochemistry in Practice" (Johnstone and Thorpe, eds., 1996; Blackwell Science), p. 69. The term "antibody" further includes single chain antibodies, CDR-grafted antibodies, chimeric antibodies, humanized antibodies, and a Fab expression library. The term also includes fusion polypeptides comprising an antibody of the invention and another polypeptide or a portion of a polypeptide (a "fusion partner"). Examples of fusion partners include biological response modifiers, lymphokines, cytokines, and cell surface antigens.

As used herein, an "antigenic determinant" is the portion of an antigen molecule, in this case a mutant apo-B100 protein, that determines the specificity of the antigen-antibody reaction. An "epitope" refers to an antigenic determinant of a polypeptide. An epitope can comprise as few as 3 amino acids in a spatial conformation which is unique to the epitope. Generally an epitope consists of at least 6 such amino acids, and more usually at least 8-10 such amino acids. Methods for determining the amino acids which make up an epitope include x-ray crystallography, 2-dimensional nuclear magnetic resonance, and epitope mapping e.g. the Pepscan method described by H. Mario Geysen et al. 1984. *Proc. Natl. Acad. Sci. U.S.A.*

81:3998-4002; PCT Publication No. WO 84/03564; and PCT Publication No. WO 84/03506.

Methods for Identifying Compounds that Affect of LDL-Proteoglycan Binding:

5 The present invention provides new assay methods for detecting, and preferably quantifying, one or more compounds that affect LDL-proteoglycan binding of interest which are present in a library of candidate compounds. Identifying compounds which inhibit LDL-proteoglycan activity is the preferred use of this assay, but it can equally be used to identify compounds which result in an increase in LDL-
10 proteoglycan activity. The terms "assay" and "assay method," as used herein, pertain to a method of detecting the presence of (*e.g.*, qualitative assay), and preferably quantifying (*e.g.*, quantitative assays), the modulation of LDL-proteoglycan binding.

 Assays of the present invention generally involve contacting the candidate compound of interest with a pre-determined non-limiting amount of both an LDL
15 reagent and a proteoglycan reagent, measuring the LDL-proteoglycan binding which results, and correlating the measured LDL-proteoglycan binding with the candidate compound's ability to affect or modulate LDL-proteoglycan binding. In a qualitative assay, simply determining whether the measured LDL-proteoglycan binding is above or below a threshold value (established, for example, using recombinant LDL
20 samples with known LDL-proteoglycan binding properties) may be sufficient to establish the assay result. Typically, when the effect is an inhibition the relationship is determined from standard samples containing known amounts of a competitive inhibitor of LDL-proteoglycan binding. Such competitive inhibitors can include, depending on the assay a non-labeled LDL or proteoglycan which has normal binding
25 activity. Thus, unless otherwise required, the term "measuring" can refer to either qualitative or quantitative determination.

 The terms "agent" or "candidate compound" as used interchangeably herein, pertain to a substance which is to be measured for a possible effect on LDL-

proteoglycan binding, preferably inhibitory activity. Candidate compounds may be inorganic or organic, though typically they are organic. Candidate compounds may be naturally occurring or synthetic. Candidate compounds are typically pharmacologically active "small molecules", but also include biological molecules such as amino acids, proteins, glycoproteins, lipoproteins, saccharides, polysaccharides, lipopolysaccharides, fatty acids, and nucleic acids. Examples of organic candidate compounds also include antibodies, antigens, haptens, enzymes, hormones, steroids, vitamins, oligonucleotides, and pharmacological agents.

The terms "sample" and "sample composition," as used herein, pertain to a composition which comprises one or more agents or candidate compounds of interest, or which may be processed to comprise one or more candidate compounds of interest. The samples used can be defined combinatorial libraries or undefined biological samples (e.g. crude plant extracts, and fungal broths). The sample or candidate compound may be in solid, emulsion, suspension, liquid, or gas form. Typically, the sample or candidate compound is processed (e.g., by the addition of a liquid buffer) so as to be a fluid (*i.e.*, free flowing) form (e.g., emulsion, suspension, solution) in order to readily permit and simplify the detection and quantification of the LDL-proteoglycan binding in the compound's presence using the methods of the invention. Typically, the sample or candidate compound of interest is present in the sample composition at a concentration of 10^{-3} M (micromolar) or less, for example, often as low as 10^{-9} M (nanomolar), sometimes as low as 10^{-12} M (picomolar), and even as low as 10^{-13} M (sub-picomolar).

The "LDL" reagent used in the assay can be any reagent which comprises at least a fragment of apo-B100 protein and maintains the LDL-proteoglycan binding activity of at least 60% of wild-type levels, preferably at least 70%, more preferably at least 80%, still more preferably 90%, most preferably at essentially 100% of wild-type LDL-proteoglycan binding activity. The apo-B100 fragment is preferably complete and preferably a wild-type human apo-B100 protein, but mutant proteins

binds to the proteoglycan to form an LDL-proteoglycan complex in the absence of said candidate compound. The appropriate conditions for such reaction mixtures are known in the art (See, e.g., Examples 8 and 10), and can in addition be determined empirically by observing whether LDL-proteoglycan complexes are formed.

- 5 Protocols may utilize a solid support to separate unbound LDL and proteoglycan reagents from LDL-proteoglycan complex, or this separation may be performed by immunoprecipitation, separation by gel electrophoresis, or affinity chromatography.

10 In one preferred embodiment, the level of LDL-proteoglycan binding is determined by gel-mobility shift assay. Prior to the assay, radiolabeled proteoglycan preparations are dialyzed. Human plasma LDL and a candidate compound are incubated with approximately 2000 dpm of (³⁵S)biglycan or (³⁵S)versican for 1 h at 37 °C. The samples are loaded into wells on agarose gel, and subjected to electrophoresis. Gels are then fixed, dried, and exposed to film. The (³⁵S)biglycan or (³⁵S)versican complexed to LDL appears as a band at the origin of the can be
15 quantitatively evaluated. This procedure has the advantages that only microgram quantities of lipoproteins are required and the relative affinity of LDL binding to the proteoglycans can be determined at physiological ionic and pH conditions.

20 In a second preferred embodiment proteoglycan-LDL binding is measured in the presence of a candidate compound as a drug screening assay. A procedure for a competitive solid-phase plate assay is employed. Normal human plasma LDL (1.0 µg in 50µl of phosphate-buffered saline (PBS) containing 0.01% EDTA per well) is immobilized by absorption to a solid support, preferably a polystyrene 96-well micrometer plates for 6 to 24 hours at 4 °C. Excess LDL is removed by washing in PBS, and nonspecific sites on the plastic are blocked by incubation with PBS
25 containing 5% bovine serum albumin (BSA) for 1 to 24 hours at 24 °C. The wells are washed three times with PBS and then with binding buffer (10 mM Tris, 50 mM NaCl, 5 mM CaCl₂, 0.05% BSA). Biotinylated proteoglycans along with a candidate compound, preferably in micromolar quantities, are added to each well and incubated

proteoglycan binding, are incubated under conditions wherein LDL binds to LDL receptor to form an LDL-LDL receptor complex in the absence of said inhibitor of LDL-proteoglycan binding. The difference between the amount of LDL-LDL receptor complex present in the mixture prepared with the candidate compound, and a control mixture prepared without the candidate compound are compared, and optionally any difference is correlated with said candidate compound's ability to affect LDL-LDL receptor binding activity.

Transgenic Animals In Vivo Model for Atherosclerosis

In addition, the present invention encompasses the use of transgenic non-human animals and mammals which express human apo-B100 as an *in vivo* model system for the study of atherosclerosis, and *in vivo* assay methods for identifying compounds which modulate atherosclerosis and/or LDL-proteoglycan binding. Identifying compounds which inhibit atherosclerosis or LDL-proteoglycan binding activity is the preferred use of this assay, but it can equally be used to identify compounds which result in an increase in atherosclerotic regions. Thus, the assays of the invention may be used to determine whether a particular food or drug composition tends to stimulate or inhibit the formation of atherosclerotic lesions. The *in vivo* assay of the invention generally involve administering a sample or candidate compound to the transgenic animal, measuring the extent of atherosclerosis or atherosclerotic lesions which results, and correlating the measured extent of atherosclerosis or atherosclerotic lesions with the candidate compound's ability to modulate atherosclerosis *in vivo*, typically by using a relationship determined from one or more control animals. In a preferred embodiment at least one of the control animals used expresses a proteoglycan receptor⁺ LDL.

In another preferred embodiment the transgenic non-human animals or mammals to which the candidate compounds or samples are administered, express a human apo-B100 protein in which the glutamine at amino acid position 2153 has

been replaced with a leucine. This mutation abolishes the formation of apo-B48, resulting in a higher yield of recombinant apo-B 100 LDL. Apo-B48 has distinct proteoglycan binding site(s) from Site B in apo-B100. Therefore use of this mutation in the *in vivo* assay methods of the invention provides an effective means for studying the portion of atherogenesis which is the result of apo-B100 mediated LDL-proteoglycan binding, as opposed to apo-B48 mediated chylomicron- and chylomicron remnant-proteoglycan binding.

In a qualitative assay, simply determining whether the measured atherosclerosis or atherosclerotic lesions is above or below a threshold value (established, for example, using recombinant LDL samples with known LDL-proteoglycan binding properties) may be sufficient to establish the assay result. Thus, unless otherwise required, the term "measuring" can refer to either qualitative or quantitative determination.

Typically, the sample or candidate compound described above for the assay for identifying agents affecting of LDL-proteoglycan binding is administered to the non-human mammal or animal, preferably a candidate compound which has demonstrated inhibition of LDL-proteoglycan binding *in vitro* is used. Preferably, the sample or candidate compound has previously been identified as a compound which affects or inhibits LDL-proteoglycan binding in one of the assays of the invention. The candidate compound can be administered by any means known in the art. Typically, the amount of sample or candidate compound of interest is controlled to deliver in a dose of 10^{-3} M (micromolar) or less, for example, often as low as 10^{-9} M (nanomolar), and sometimes as low as 10^{-12} M (picomolar) in the subject animal's plasma.

The animal used can be any non-human animal preferably a mammal, more preferably a primate, rabbit, pig, goat or rodent, still more preferably a mouse, most preferably the recombinant control mouse described in Example 3. The animal must express a human apo-B100 protein, or at least a sufficiently large fragment thereof to

allow the animal's recombinant LDL to bind to endogenous arterial wall, which can be predicted by demonstrating LDL-proteoglycan binding in an *in vitro* assay (See, e.g., Example 8).

In addition, the non-human transgenic animal must be subjected to conditions wherein measurable atherosclerotic lesions form in the arteries of said animal. As used herein the phrase "conditions wherein measurable atherosclerotic lesions form in the arteries of said animal" is used to denote any conditions which are known to cause atherosclerotic lesions in the particular animal used in an experiment. These conditions are particular to the animal used and must be determined empirically to ensure that the lesions are in fact measurable by whatever method is used. Most of such conditions relate to the diet of the animal or to the animal's genetic make up. With respect to diet, cholesterol, cholesteryl ester, bile salts and fats, particularly saturated fats are known to induce atherosclerotic lesions when consumed in high doses. The Paigen diet described in Example 9 is an example of such a dietary condition. In terms of genetics, factors such as defective LDL receptors, mutant apolipoprotein genes, particularly apo-B100, which disrupt LDL/LDL receptor binding are example of genetic conditions.

The amount of atherosclerosis or atherosclerotic lesions is measured by many methods known in the art including the morphometric imaging method described in Example 9, as well as arteriography and ultrasound. The difference between the extent of atherosclerosis or atherosclerotic lesions present in the animal which has been administered the test compound and a control animal which has not received the test compound is determined. Preferably the control animal is precisely the same type and strain of animal and that which received the candidate compound, and has been treated with the same conditions.

Mutant apo-B100 Proteins, Fragments and LDL:

5 The invention embodies polypeptides comprising a proteoglycan⁻receptor⁺ Site B, and entire apo-B100 proteins comprising a proteoglycan⁻receptor⁺ Site B, as well as fragments thereof which comprise a proteoglycan⁻receptor⁺ Site B flanked on at least one side by a contiguous sequence of at least N amino acids which is directly adjacent to Site B in the wild-type human apo-B100 sequence, where the number N is about 25 amino acids, preferably 20 amino acids, more preferably 15 amino acid, still more preferably 8 to 10 amino acids, most preferably 6 amino acids. The apo-B100 proteins of the invention also comprise proteins that have a leucine in place of glutamine at amino acid position 2153, which abolishes the formation of apo-B48, resulting in a higher yield of recombinant apo-B 100 LDL.

10 The proteins of the invention can be made using routine expression methods known in the art. The DNA encoding the desired polypeptide, may be ligated into an expression vector suitable for any convenient host. Both eukaryotic and prokaryotic host systems may be used in forming recombinant polypeptides, and a summary of some of the more common systems are included below in the description of expression vectors. The polypeptide is then isolated from lysed cells or from the culture medium and purified to the extent needed for its intended use. Purification may be by any technique known in the art, for example, differential extraction, salt fractionation, chromatography, centrifugation, and the like. See, for example, *Methods* in Enzymology for a variety of methods for purifying proteins.

15 In addition, shorter protein fragments may be produced by chemical synthesis. Alternatively the proteins of the invention may be extracted from recombinant LDL. Methods for purifying apolipoproteins, particularly apo-B100 are known in the art, and include the use of detergents or chaotropic agents to disrupt particles followed by differential extraction and separation of the apo-B100 proteins and lipids by ion exchange chromatography, affinity chromatography, sedimentation according to density, and gel electrophoresis. Recombinant LDL can be isolated from a transgenic

animal as described in Examples 3 and 4. The term recombinant LDL also embraces reconstituted LDL as well as LDL derived from a transgenic non-human animal, as described above. Methods of reconstituting LDL are known in the art. See, for example, Corsini, A. et al. 1987. *J. Lipid Res.* 28:1410-1423. Such reconstituted LDL may comprise lipids from solely human sources, as well as lipids and labels which are not naturally associated with LDL. Such reconstituted recombinant LDL must, however, comprise a mutant human apo-B100 protein of the invention.

As used herein, the term "purified recombinant LDL" refers to a recombinant LDL which is essentially free, i.e., contains less than about 50%, preferably less than about 30%, and more preferably less than about 10%, even more preferably less than about 5%, and still more preferably less than about 1% of lipoproteins comprising one or more non-human apolipoproteins. Methods for purifying recombinant LDL include centrifugation to separate lipoproteins of a particular density from other plasma constituents, as well as affinity chromatography utilizing an antibody which is specific for an antigenic determinant found only on the recombinant LDL or only on the other lipoproteins produced by a transgenic non-human animal.

The present invention also encompasses non-LDL lipoprotein particles which comprise an apo-B100 protein or fragment of the invention. These lipoproteins may include other human apolipoproteins which are normally associated with apo-B100 (e.g. apo-E and possibly apo-C), and may have the same rough physical properties of VLDL or IDL. They may also include apo-lipoproteins which are native to the transgenic animal from which they are isolated. These non-LDL lipoproteins particles can be isolated and used as a source of purified mutant apo-B100 protein.

Antibodies to Proteoglycan Receptor⁺ Mutant apo-B100 Proteins and LDL:

Apo-B100 proteins comprising proteoglycan receptor⁺ mutations, fragments thereof comprising Site B, and recombinant LDL particles comprising either of these apo-B100 proteins or fragments are used to produce antibodies, including both

polyclonal and monoclonal. If polyclonal antibodies are desired, a suitable non-human animal, preferably a non-human mammal is selected, usually a mouse, rat, rabbit, goat, or horse, is immunized with an apo-B100 protein, fragment, or LDL comprising the proteoglycan receptor⁺ Site B in the presence of an appropriate adjuvant (e.g. aluminum hydroxide, RIBI, etc.) which is known in the art. In addition the protein, fragment or LDL can be pretreated with an agent which will increase antigenicity, such agents are known in the art and include, for example, methylated bovine serum albumin (mBSA), bovine serum albumin (BSA), Hepatitis B surface antigen, and keyhole limpet hemocyanin (KLH). Serum from the immunized animal is collected, treated and tested according to known procedures. If the serum contains polyclonal antibodies to undesired epitopes, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art, see for example, Mayer and Walker (1987).

Alternatively, monoclonal antibodies directed against an apo-B100 protein, fragment, or LDL comprising the proteoglycan receptor⁺ Site B can also be readily produced by one of ordinary skill in the art. The general methodology for making monoclonal antibodies by hybridoma is well known. Immortal antibody-producing cell lines can be created by cell fusion. See, for example, Harlow, E., and D. Lane. 1988. Antibodies A Laboratory Manual. Cold Spring Harbor Laboratory. pp. 53-242.

Transgenic non-human animals or mammals which express a human apo-B100 LDL with a wild-type Site B amino acid sequence are particularly useful for preparing antibodies, as these animals will recognize all or most of the exposed regions of human apo-B100 as self antigens. Thus, when such an animal is exposed, for example, to an LDL particle comprising an apo-B100 with a proteoglycan receptor⁺ Site B, this Site B will be one of the few if not the only new immunogenic site exposed, and antibodies to this mutant site will be preferentially produced. Alternatively, the antibodies of the invention can be screened by standard ELISA

technique for there ability to bind to apo-B100 with a proteoglycan⁻receptor⁺ Site B, while not binding to a recombinant control LDL.

Antibodies, both monoclonal and polyclonal, which are directed to a proteoglycan⁻receptor⁺ Site B are useful for screening for the presence of such mutations in the population at large with standard RIA and ELISA assay techniques. In addition these antibodies are may be used to purify the a proteoglycan⁻receptor⁺ recombinant LDL by affinity chromatography.

Polynucleotides, Cells, and Transgenic Animals:

The invention embodies polynucleotides which encode a polypeptide comprising a proteoglycan⁻receptor⁺ Site B, and entire apo-B100 proteins comprising a proteoglycan⁻receptor⁺ Site B, as well as fragments thereof which comprise a proteoglycan⁻receptor⁺ Site B flanked on at least one side by a contiguous sequence of at least N amino acids which is directly adjacent to Site B in the wild-type human apo-B100 sequence, where the number N is about 25 amino acids, preferably 20 amino acids, more preferably 15 amino acids, still more preferably 8 to 10 amino acids, most preferably 6 amino acids. Generally the polynucleotides of the invention comprise the naturally occurring nucleotide sequence for the portions of the gene which encode the amino acid sequences outside of Site B as shown in the apo-B100 gene sequence of Knott, T, et al. 1986. *Nature* 323:734-738. However, any naturally occurring silent codon variation or other silent codon variation can be employed to encode those amino acids outside of Site B. Similarly those nucleotide sequences which encode the portions of Site B which maintain the wild-type apo-B100 sequence will generally make use of the naturally occurring nucleotide sequence, but any naturally occurring silent codon variation or other silent codon variation can be employed. As for those amino acids which are changed or added to the proteoglycan⁻receptor⁺ Site B, nucleic acid sequences generally will be chosen to optimize

expression in the specific human or non-human animal in which the polynucleotide is intended to be used, making use of known codon preferences.

The nucleic acids of the invention include expression vectors, amplification vectors, PCR-suitable polynucleotides, and vectors which are suitable for the introduction of a polynucleotide of the invention into an embryonic stem cell for the production of transgenic non-human animals. In addition, vectors which are suitable for the introduction of a polynucleotide of the invention into cells, organs and individuals, including human individuals, for the purposes of gene therapy to reduce the severity of or prevent atherosclerosis are encompassed. The invention also encompasses targeting vectors and method for changing a wild-type Site B into a proteoglycan receptor⁺ Site B in a human apo-B100 gene contained within an embryonic stem cell.

The invention embodies amplification vectors, which comprise a polynucleotide of the invention, and an origin of replication. Preferably, such amplification vectors further comprise restriction endonuclease sites flanking the polynucleotide, so as to facilitate cleavage and purification of the polynucleotides from the remainder of the amplification vector, and a selectable marker, so as to facilitate amplification of the amplification vector. Most preferably, the restriction endonuclease sites in the amplification vector are situated such that cleavage at those site would result in no other amplification vector fragments of a similar size.

Thus, such an amplification vector may be transfected into a host cell compatible with the origin of replication of said amplification vector, wherein the host cell is a prokaryotic or eukaryotic cell, preferably a mammalian, insect, yeast, or bacterial cell, most preferably an *Escherichia coli* cell. The resulting transfected host cells may be grown by culture methods known in the art, preferably under selection compatible with the selectable marker (e.g., antibiotics). The amplification vectors can be isolated and purified by methods known in the art (e.g., standard plasmid prep procedures). The polynucleotide of the invention can be cleaved with restriction

enzymes that specifically cleave at the restriction endonuclease sites flanking the polynucleotide, and the double-stranded polynucleotide fragment purified by techniques known in the art, including gel electrophoresis.

Alternatively linear polynucleotides comprising a polynucleotide of the invention may be amplified by PCR. The PCR method is well known in the art and described in, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202 and Saiki, R et al. 1988. *Science* 239:487-491, and European patent applications 86302298.4, 86302299.2 and 87300203.4, as well as *Methods in Enzymology* 1987 155:335-350.

The polynucleotides of the invention also include expression vectors. Expression vector systems, control sequences and compatible host are known in the art. For a review of these systems see, for example, U.S. Patent No. 5,350,671, columns 45-48.

The polynucleotides of the invention can also be derivatized in various ways, including those appropriate for facilitating transfection and/or gene therapy. The polynucleotides can be derivatized by attaching a nuclear localization signal to it to improve targeted delivery to the nucleus. One well-characterized nuclear localization signal is the heptapeptide PKKKRKV (pro-lys-lys-lys-arg-lys-val). Preferably, in the case of polynucleotides in the form of a closed circle, the nuclear localization signal is attached via a modified loop nucleotide or spacer that forms a branching structure.

If it is to be used *in vivo*, the polynucleotide of the invention may be derivatized to include ligands and/or delivery vehicles which provide dispersion through the blood, targeting to specific cell types, or permit easier transit of cellular barriers. Thus, the polynucleotides of the invention may be linked or combined with any targeting or delivery agent known in the art, including but not limited to, cell penetration enhancers, lipofectin, liposomes, dendrimers, DNA intercalators, and nanoparticles. In particular, nanoparticles for use in the delivery of the polynucleotides of the invention are particles of less than about 50 nanometers diameter, nontoxic, non-antigenic, and comprised of albumin and surfactant, or iron

as in the nanoparticle particle technology of SynGenix. In general the delivery vehicles used to target the polynucleotides of the invention may further comprise any cell specific or general targeting agents known in the art, and will have a specific trapping efficiency to the target cells or organs of from about 5 to about 35%.

5 The polynucleotides of the invention may be used *ex vivo* in a gene therapy method for obtaining cells or organs which produce proteoglycan receptor⁺ LDL. The cells are created by incubation of the target cell with one or more of the above-described polynucleotides under standard conditions for uptake of nucleic acids, including electroporation or lipofection. In practicing an *ex vivo* method of treating
10 cells or organs, the concentration of polynucleotides of the invention in a solution prepare to treat target cells or organs is from about 0.1 to about 100 μ M, preferably 0.5 to 50 μ M, most preferably from 1 to 10 μ M.

Alternatively, the oligonucleotides can be modified or co-administered for targeted delivery to the nucleus. Improved oligonucleotide stability is expected in the
15 nucleus due to: (1) lower levels of DNases and RNases; and (2) higher oligonucleotide concentrations due to lower total volume.

Alternatively, the polynucleotides of the invention can be covalently bonded to biotin to form a biotin-polynucleotide prodrug by methods known in the art, and co-administered with a receptor ligand bound to avidin or receptor specific antibody
20 bound to avidin, wherein the receptor is capable of causing uptake of the resulting polynucleotide-biotin-avidin complex into the cells. Receptors that cause uptake are known to those of skill in the art. Any transplantable cell type or organ can be used preferably hepatic cells, fetal hepatic cells or whole or partial livers.

The invention encompasses vectors which are suitable for the introduction of
25 a polynucleotide of the invention into an embryonic stem cell for the production of transgenic non-human animals, which in turn result in the expression of recombinant LDL in the transgenic animal. The size of the apo-B100 dictates that a vector which can accommodate inserts which are tens of thousands of bases long. Yeast artificial

chromosomes (YAC), bacterial artificial chromosomes (BAC), bacteriophage P1, and other vectors known in the art which are able to accommodate sufficiently large inserts to encode the entire apo-B100 gene. The 95-kb apo-B P1 plasmid p158 of Linton, M. et al. 1993. *J. Clin. Invest.* 92:3029-3037 makes a convenient vector system to use, as it already contains a full length apo-B100 gene.

Moreover, Borén, J. et al. 1996. *Genome Res.* 6:1123-1130 have demonstrated how to isolate a 5.7 kb fragment of the apo-B100 gene which comprises Site B, in order to perform site-directed mutagenesis as described below in Examples 1 and 2, using RARE cleavage. In brief, RecA-assisted restriction endonuclease (RARE) cleavage consists of protecting a specific restriction endonuclease site with a complementary oligonucleotide. In the presence of RecA, a triplex DNA complex is formed that prevents methylation at the protected sites, for example *EcoRI*-35763 and *EcoRI*-41496 were protected by oligonucleotides (5' gaaaactcccacagcaagctaattgattatctgaattcattcaattgggagagacaa gtttcac 3') and (5' cacaagtgaatatctggttaggtagaattctcccagtttcacaatgaaaacatc 3') respectively, while unprotected sites are methylated by the corresponding methylase. After dissociation of the oligonucleotides, the protected sites can be cleaved with the restriction endonuclease which corresponds to the protected sites, for example *EcoRI*. All of the non-protected *EcoRI* site had been methylated and were thus not subject to cleavage by the restriction enzyme. The resulting fragment of the apo-B100 gene can then be ligated into a smaller vector which is appropriate for site-directed mutagenesis, e.g. pZER0. The site-directed mutagenesis process is then conducted by techniques well known in the art, and the fragment is return and ligated to the larger vector from which it was cleaved. For site directed mutagenesis methods see, for example, Kunkel, T. 1985. *Proc. Natl. Acad. Sci. U.S.A.* 82:488; Bandeyar, M. et al. 1988. *Gene* 65: 129-133; Nelson, M., and M. McClelland 1992. *Methods Enzymol.* 216:279-303; Weiner, M. 1994. *Gene* 151: 119-123; Costa, G. and M. Weiner. 1994.

Nucleic Acids Res. 22: 2423; Hu, G. 1993. *DNA and Cell Biology* 12:763-770; and Deng, W. and J. Nickoff. 1992. *Anal. Biochem.* 200:81.

Briefly, the transgenic technology used herein involves the inactivation, addition or replacement of a portion of a gene or an entire gene. For example the present technology includes the addition of human proteoglycan⁻receptor⁺ apo-B100 genes with or without the inactivation of the non-human animal's native apolipoprotein genes, as described in the preceding two paragraphs and in the Examples. The invention also encompasses the use of vectors, and the vectors themselves which target and modify an existing human apo-B100 gene in a stem cell, whether it is contained in a non-human animal cell where it was previously introduced into the germ line by transgenic technology or it is a native apo-B100 gene in a human pluripotent cell. This transgene technology usually relies on homologous recombination in a pluripotent cell that is capable of differentiating into germ cell tissue. A DNA construct that encodes an altered region of comprising a proteoglycan⁻receptor⁺ Site B or an altered region of the non-human animal's apolipoprotein gene the contains, for instance a stop codon to destroy expression, is introduced into the nuclei of embryonic stem cells. Preferably mice are used for this transgenic work. In a portion of the cells, the introduced DNA recombines with the endogenous copy of the cell's gene, replacing it with the altered copy. Cells containing the newly engineered genetic alteration are injected in a host embryo of the same species as the stem cell, and the embryo is reimplanted into a recipient female. Some of these embryos develop into chimeric individuals that posses germ cells entirely derived from the mutant cell line. Therefore, by breeding the chimeric progeny it is possible to obtain a new strain containing the introduced genetic alteration. See Capecchi 1989. *Science*. 244:1288-1292 for a review of this procedure.

The present invention encompasses the polynucleotides described herein, as well as the methods for making these polynucleotides including the method for creating a

mutation in a proteoglycan receptor⁺ mutation in a human apo-B100 gene. In addition, the present invention encompasses cells which comprise the polynucleotides of the invention, including but not limited to amplification host cells comprising amplification vectors of the invention. Furthermore the present invention comprises the embryonic stem cells and transgenic non-human animals and mammals described herein which
5 comprise a gene encoding a proteoglycan receptor⁺ human apo-B100 protein.

The invention also encompasses methods for preventing or reducing the severity of atherosclerosis in an animal or mammal, by expressing a polynucleotide encoding a proteoglycan receptor⁺ human apo-B100 protein or protein fragment of the invention. The polynucleotide encoding a proteoglycan receptor⁺ human apo-B100 protein or protein fragment of the invention is transduced into a cell, either *ex vivo* or *in situ*. In the case of *ex vivo* transduction, the transduced cell is then administered to an animal or mammal. Expression of the proteoglycan receptor⁺ human apo-B100 protein or protein fragment of the invention substantially prevents, ameliorates or reduces the severity of atherosclerosis in the animal or mammal.
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The polynucleotide to be transduced is normally inserted into an appropriate expression vector, using standard molecular biology techniques. Appropriate expression vectors are easily selected by one of skill in the art, and generally include *cis*-acting transcription and translation nucleotide sequences which are operable in the cell to be transduced. Such elements are well known in the art, and include viral promoters and enhancers (*e.g.*, the SV40 promoter and enhancer), mammalian constitutive promoters (*e.g.*, the β -actin promoter), mammalian tissue-specific promoters and enhancers, polyadenylation signals, and the like. Preferably, an intron is introduced into the polynucleotide encoding the proteoglycan receptor⁺ human apo-B100 protein or protein fragment, as the presence of an intron frequently improves mRNA processing and export from the nucleus.
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case, an effective amount of transduced cells (*i.e.*, an amount sufficient to prevent, ameliorate, or reduce atherosclerosis in the subject) are transferred to the subject.

Methods of *ex vivo* transduction are well known in the art. See, for example, U.S. Patent No. 5,399,346. Viral transduction is also well known, and is disclosed in a number of issued U.S. patents, such as U.S. Patents Nos. 5,672,344, 5,656,465, 5,139,941, and 5,851,529. Transduction of target cells with naked DNA technology is disclosed in, for example, U.S. Patent No. 5,580,859. The quantity of the polynucleotide of the invention administered to the animal or mammal subject by *in situ* transduction will vary according to a number of parameters, such as the efficiency of the transduction method, the desired levels of expression, the subject's medical history, and other parameters known to one of skill in the art.. In any case, an effective amount of a polynucleotide of the invention (*i.e.*, an amount sufficient to prevent, ameliorate, or reduce atherosclerosis in the subject) is administered to the subject.

As will be apparent to one of skill in the art, blood levels of proteoglycan⁻ receptor⁺ human apo-B100 protein or protein fragment may be measured after *in situ* transduction or *ex vivo* transduction and transfer of transduced cells to the subject. If blood levels of the protein or protein fragment are not at desired levels, transduction or transduction and transfer may be repeated to achieve the desired levels of the protein or protein fragment of the invention.

After transduction (for *in situ* transduction) or transduction and transfer to the animal or mammal (for *ex vivo* transduction), expression of the polynucleotide encoding a proteoglycan⁻ receptor⁺ human apo-B100 protein or protein fragment results in prevention of or a reduction or amelioration of the severity of symptoms of atherosclerosis.

Throughout this application, various publications, patents, and published patent applications are cited. The disclosures of the publications, patents, and published patent specifications referenced in this application are hereby incorporated

by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

EXAMPLES

Several of the methods of the present invention are described in the following examples, which are offered by way of illustration and not by way of limitation. Many other modifications and variations of the invention as herein set forth can be made without departing from the spirit and scope thereof and therefore only such limitations should be imposed as are indicated by the appended claims.

Example 1

Generation of Truncated P1 Plasmids and Isolation of DNA Fragments for Mutagenesis.

The 95-kb apo-B P1 plasmid p158 (Linton, M. et al. 1993. *J. Clin. Invest.* 92:3029-3037) was prepared and modified by RARE cleavage as described by Borén, J. et al. 1996. *Genome Res.* 6:1123-1130. Oligomers *EcoRI*-35763 (5' gaaaactcccacagcaagctaataatgattatctgaattcattcaattgggagagacaagtttcac 3') and *EcoRI*-41496 (5' cacaagtgaatatctggtaggatagaattctcccagtttcacaatgaaaacatc 3') were used to make 5.7-kb-deleted P1 plasmid. A 5.7-kb fragment was isolated from the apo-B100 "Leu-Leu" plasmid with RARE cleavage using oligomers *EcoRI*-35763 and *EcoRI*-41496 and cloned into the pZER0-1 vector (Invitrogen). The apo-B100 "Leu-Leu" plasmid was used to increase the yield of apo-B100, since it contains a CAA to CTA mutation in codon 2153 that effectively abolished the formation of apo-B48. The latter of which is formed by an editing mechanism present in mouse livers (Yao, Z. et al. 1992. *J. Biol Chem.* 267:1175-1182).

Example 2

Site-Directed Mutagenesis of P1 DNA.

The pZER0-5.7 kb plasmid was subjected to site-directed mutagenesis with the Morph System (5 Prime→3 Prime, Inc.®) using oligonucleotide K3363E (5' caagattgacaagagaaaggggattgaag 3') to mutate the lysine at residue 3363 to glutamic acid. The resulting plasmids were subjected to RARE cleavage with oligomers *EcoRI*-35763 and *EcoRI*-41496, and the mutated 5.7-kb fragment was isolated. After RARE cleavage of the 5.7-kb-deleted P1 plasmid with oligonucleotide *EcoRI* del. 5.7-kb (5' ggaaaactccacagcaagctaagtattctgaattctccc agtttcacaatgaaaacatc 3'), the mutated 5.7-kb fragment was ligated into the linearized and phosphatased 5.7-kb-deleted P1 vector (Borén, J. et al. 1996. *Genome Res.* 6:1123-1130).

Example 3

Human Apo-B Transgenic Mice.

The transgenic mice were generated with a P1 bacteriophage clone (p158) (Linton, M. et al. 1993. *J. Clin. Invest.* 92:3029-3037) that spanned the human apo-B gene in which mutations had been introduced by RecA-assisted restriction endonuclease (RARE) cleavage (Borén, J. et al. 1996. *Genome Res.* 6:1123-1130) as described in Examples 1 and 2.

P1 DNA was prepared and microinjected into fertilized mouse eggs (C57BL/6 x SJL) (McCormick, S. et al. 1994. *Genet Anal Tech Appl* 11:158-164). Mice were housed in a pathogen-free barrier facility operating on a 12-h light/12-h dark cycle and were fed rodent chow containing 4.5% fat (Ralston Purina, St. Louis, MO).

Transgenic mice were identified at the time of weaning (21 days) by screening mouse plasma for human apo-B 100 western dot-blot and western analysis with the monoclonal antibody 1D1 (Milne, R. et al. 1983. *Arteriosclerosis.* 3:23-30). Four

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different types of human recombinant LDL were generated (Table 1). The first of the transgenic mouse lines expressed recombinant control LDL. This LDL, however, had a leucine in place of glutamine at amino acid position 2153, which abolishes the formation of apo-B48, resulting in a higher yield of recombinant apo-B 100 LDL. Its receptor-binding activity was found to be identical to that of LDL generated by the unmodified apo-B 100 P1 bacteriophage clone. The second transgenic mouse line expressed a form of recombinant LDL that had a single amino acid mutation, the substitution of glutamine for the normally occurring arginine at residue 3500 in apo-B100 (R3500Q). We have also proven that this mutation is identical to the mutation that causes defective receptor binding in the genetic disorder familial defective apo-B 100 (Borén, J. et al. 1998. *J. Clin. Invest.* 101:1084-1093). Although it is outside the receptor-binding site (Site B), this mutation produces a conformational change that disrupts receptor binding. This is the only LDL that did not have the "Leu-Leu" mutation encoded in the apo-B mRNA. The third transgenic mouse line expressed a recombinant LDL in which the basic amino acids in Site B (residues 3359-3369) were converted to neutral amino acids. The arginine residues were converted to serines and the lysine residues to alanines (RK3359-3369SA). These changes virtually abolish the receptor-binding activity of the recombinant LDL; this finding along with other evidence demonstrated that Site B is the receptor-binding site of LDL (Borén, J. et al. 1998. *J. Clin. Invest.* 101:1084-1093). The fourth transgenic mouse line expressed human recombinant LDL in which the lysine at residue 3363 of apo-B 100 was changed to glutamic acid (K3363E). This mutation was designed to disrupt proteoglycan binding if Site B plays a significant role in binding to proteoglycans.

Table 1. Mutants of the Human Apo-B Gene

Recombinant LDL	LDL Receptor binding	Proteoglycan binding
Control LDL	Normal	Normal
R3500Q LDL	Defective	Normal
RK3359-3369SA LDL	Defective	Defective
K3363E LDL	Normal	Defective

Example 4

Isolation of Recombinant Lipoproteins.

Blood from mice fasted for 5 h was collected by cardiac puncture into tubes containing EDTA (final concentration 1 mg/ml), and the plasma was mixed with butylated hydroxytoluene (final concentration, 25 μ M), phenylmethyl sulfonylfluoride (final concentration, 1 mM), and aprotinin (final concentration, 10 U/ml). The LDL ($d=1.02-1.05$ g/ml) were isolated by sequential ultracentrifugation (Ti 70 rotor) and dialyzed against 150 mM NaCl and 0.01% EDTA, pH 7.4, and the mouse apo-E and apo-B were removed by immunoaffinity chromatography. The $d = 1.02-1.05$ g/ml fraction was mixed with an equal volume (850 μ l) of AffiGel Hz (BioRad) and incubated for 17 hours at 4 °C in a gently rocking tube filled with nitrogen. The AffiGel Hz (100g) had previously been coupled with mouse apo-E or mouse apo-B rabbit immunoglobulins from 50 ml of antiserum. Lipoproteins used for receptor-binding experiments were isolated and assayed within 1 week. Human plasma LDL, isolated from a single blood donor, were included as a control in each experiment.

The recombinant LDL were isolated from the human apo-B transgenic mouse plasma by ultracentrifugation, and the endogenous mouse apo-E- and apo-B-containing lipoproteins were removed by immunoaffinity chromatography. The

purified lipoproteins were analyzed on western blots of 3-15% polyacrylamide-SDS gels with ECL western blotting detection reagents (Amersham). Purified recombinant LDL isolated from the plasma of the four lines of transgenic mice contained intact apo-B100 without any visible contamination (Fig. 1A). Western blot analysis showed that the recombinant LDL from all four transgenic mouse lines bound to the monoclonal antibody 1D1 (Fig. 1B), whose epitope is between amino acids 474 and 539 in human apo-B100 (Milne, R. et al. 1983. *Arteriosclerosis*. 3:23-30) and that only the unpurified recombinant LDL reacted with polyclonal antibodies against mouse apo-B and mouse apo-E (Fig. 1C and Fig. 1D, respectively), confirming that endogenous mouse apo-B and apo-E had been completely removed.

Example 5

Modification Of Recombinant LDL.

To selectively modify arginines or lysines in apo-B100, recombinant LDL were incubated with acetic anhydride or cyclohexadione, respectively. Acetylation of LDL was carried out as described by Basu, S. et al. 1976. *Proc. Natl. Acad Sci. USA*. 73:3178-3182. In short, with continuous stirring in ice water bath, recombinant LDL (0.5 mg) in 1.0 ml 0.15 M NaCl and 0.01 % EDTA were mixed with 1.5 μ l saturated sodium acetate solution every 15 min over 1 hr. Cyclohexanedione modification of LDL was performed as by Mahley, R. et al. 1977. *J. Biol Chem* 252:7279-7287. Recombinant LDL (0.5 mg) in 1 ml of 0.15 M NaCl and 0.01 % EDTA was mixed with 2 ml of 0.15 M 1,2-cyclohexanedione in 0.2 M sodium borate buffer (pH 8.1) and incubated at 35 °C for 2 h. The sample was then dialyzed for 48 h against 0.15 M NaCl and 0.01% EDTA at 4 °C.

Example 6

Cell Culture and Competitive Receptor Binding Assay.

Human fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Seven days before each experiment, the fibroblasts were plated into 12-well cell culture dishes (22-mm diameter per well) at ~ 12000 cells/well in the same medium. Two days before each experiment, the cells were transferred to DMEM containing 10% human lipoprotein-deficient serum. Normal human ^{125}I -labeled LDL (2 $\mu\text{g/ml}$) along with increasing concentrations of unlabeled lipoproteins were added to the cells in DMEM containing 25 mM HEPES and 10% human lipoprotein-deficient serum. After a 3-h incubation at 4° C, the surface-bound radioactivity was determined. The amount of unlabeled lipoproteins needed to compete 50% with ^{125}I -labeled LDL was determined from an exponential decay curve-fitting model (Arnold, K. et al. 1992. *Lipoprotein Analysis. A Practical Approach*. C. A. Converse, and E. R. Skinner, editors. Oxford University Press, Oxford. 145-168).

To evaluate the receptor-binding activity of the recombinant LDL, LDL from each transgenic line were tested with an in vitro competitive receptor-binding assay (Fig. 2). Recombinant LDL with the uncharged Site B (RK3359-3369SA) or with the R3500Q mutation had defective receptor binding ($\text{ED}_{50} > 20 \mu\text{g/ml}$ for both), a finding in agreement with other results obtained in our lab (Borén, J. et al. 1998. *J. Clin. Invest.* 101:1084-1093). The K3363E LDL had normal receptor binding equivalent to that of control LDL (ED_{50} 2.4 and 2.3 $\mu\text{g/ml}$, respectively). Moreover, since LDL with the K3363E mutation retained LDL receptor-binding activity, these results also indicate that the mutation did not affect the overall folding and stability of the protein.

Example 7

Biglycan And Versican Isolation.

Biglycan and versican were prepared from cultured human arterial smooth muscle cells metabolically labeled with (^{35}S) SO_4 , as described previously (Chang, Y. et al. 1983. *J. Biol. Chem.* 258:5679-5688). Briefly, cell medium was concentrated on DEAE-Sephacel minicolumns equilibrated in 8 M urea, 0.25 M NaCl, and 0.5% CHAPS. The (^{35}S)labeled proteoglycans were eluted with 8 M urea, 3 M NaCl, and 0.5% CHAPS and applied to a Sepharose CL-2B column equilibrated in 8 M urea and 0.5% CHAPS. Small aliquots of the resulting fractions were counted to provide a profile of the separated material. The fractions were then combined into four pools: pool 1, $K_{av} = 0.2$; pool 2, $K_{av} = 0.2-0.4$; pool 3, $K_{av} = 0.4-0.55$; and pool 4; $K_{av} = 0.55-0.8$. Eluted material in each pool was concentrated on Centricon-50 spin columns and dialyzed into Buffer A used for binding assays. The bulk of the (^{35}S) SO_4 radioactivity was present in pools 1 and 3. Western blot analyses showed that pool 1 contained versican and was negative for perlecan, biglycan, and decorin. Pool 3 contained only biglycan and no immunoreactivity for perlecan, versican, or decorin. Only very small amounts of decorin were found in pool 4.

Example 8

Gel-Mobility Shift Assay

The interaction between LDL and biglycan or versican was investigated by a gel-mobility shift assay (Camejo, G. et al. 1993. *J. Biol Chem.* 268:14131-14137). Before the assay, the (^{35}S)biglycan and (^{35}S)versican preparations were dialyzed extensively at 4 °C against 10 mM HEPES, 150 mM NaCl, 5 mM CaCl_2 , and 2 mM MgCl_2 (pH 7.4, Buffer A), and the protein concentrations were determined (BioRad Laboratories) with bovine gamma globulin as the standard. Increasing concentrations of LDL were incubated with approximately 2000 dpm of (^{35}S)biglycan or (^{35}S)versican for 1 h at 37 °C in a total volume of 20 μl of Buffer A. Three microliters

of bromophenol blue:glycerol (1:1, v/v) was added to the samples, and 20 μ l was loaded into wells of 0.7% NuSieve (FMC BioProducts) agarose gels prepared on Gel-Bond film (FMC BioProducts). Electrophoresis was run for 3 h at 60V with recirculating buffer (10 mM HEPES, 2 mM CaCl_2 , 4 mM MgCl_2 pH 7.2) in a cold room. Gels were fixed with 0.1 % cetyl pyridium chloride in 70% ethanol for 90 min, air-dried, and exposed to Hyper Film-MP (Amersham Life Sciences) at -70°C . The (^{35}S)biglycan or (^{35}S)versican complexed to LDL appears as a band at the origin of the were quantitatively evaluated with a Hewlett Packard Scan Jet II cx and ImageQuant software (Molecular Dynamics).

Gel-shift analysis of recombinant LDL with versican and biglycan.

To determine the ability of the different recombinant LDL to interact with proteoglycans, recombinant control, R3500Q, RK3359-3369SA, and K3363E LDL were isolated and subjected to gel-shift analysis (Camejo, G. et al. 1993. *J. Biol Chem.* 268:14131-14137). This procedure has the advantages that only microgram quantities of lipoproteins are required and the relative affinity of LDL binding to the proteoglycans can be determined at physiological ionic and pH conditions. In three independent experiments, recombinant control LDL and R3500Q LDL bound (^{35}S)versican and (^{35}S)biglycan almost as efficiently as human plasma LDL, but recombinant RK3359-3369SA and K3363E LDL had severely impaired binding to both (^{35}S)versican and (^{35}S)biglycan (Fig. 3). Thus, mutations of basic amino acids in Site B dramatically reduced the ability of apo-B100 to interact with proteoglycans. Of particular interest was that recombinant K3363E LDL interacted defectively with both versican and biglycan but had normal receptor binding. Recombinant control LDL with and without the "Leu-Leu" mutation displayed identical binding to (^{35}S)versican and (^{35}S)biglycan (data not shown). Thus, this mutation does not affect the binding of LDL to proteoglycans.

Gel-shift analysis of acetylated or cyclohexanedione-modified recombinant LDL.

Mutagenesis of Site B severely reduced its interaction with versican and biglycan. To test the importance of the remaining clusters of basic amino acids for the interaction of LDL with versican or biglycan, the remaining arginines or lysines in apo-B100 were selectively modified to abolish the receptor-binding and heparin-binding activities of LDL. Recombinant LDL isolated from human apo-B transgenic mice expressing recombinant control or RK3359-3369SA LDL were divided into three aliquots. Two aliquots were selectively modified with acetic anhydride or cyclohexanedione to change the arginines and lysines, respectively. The ability of the modified recombinant control or RK3359-3369SA LDL to bind (35 S)biglycan or (35 S)versican was compared with that of unmodified recombinant LDL by gel-shift analysis. Again, unmodified RK3359-3369SA LDL had greatly reduced ability to interact with (35 S)biglycan or (35 S)versican (Fig. 4). Furthermore, the unmodified RK3359-3369SA LDL bound proteoglycans almost identically before and after chemical modification; only a minor difference was detected in the (35 S)biglycan gel-shift assay (Fig. 4A). These data demonstrate that Site B is the most important functional site for interaction with proteoglycans and that the seven other potential sites do not play a significant role.

Example 9
Initial *In Vivo* Atherosclerosis Studies
Using Proteoglycan-Binding-Defective LDL

These studies were designed to determine if elevated levels of proteoglycan-binding-defective LDL would be less atherogenic than similar levels of wild-type recombinant LDL. The RK3359-3369SA construct was used to generate mice expressing the proteoglycan-binding-defective LDL. The apo-B transgenic mice used in this atherosclerosis study were hybrids of the genetic strains C57BL/6 (50%) and

SJL (50%). Non-transgenic mice with the same genetic background were also included in the study. The transgenic mice were fed a Paigen diet containing 1.2% cholesterol, 0.5% bile salts, and 20% fat for 17 weeks. The mice were then sacrificed, and the aortas were perfusion fixed and analyzed with the *en face* procedure, in which the entire aorta is pinned out flat, stained with Sudan IV, and analyzed with a morphometric image-analysis system (Image-1/AT) to quantitate the extent of atherosclerosis.

In both groups of transgenic mice, the percentage of the vessel wall covered by atherosclerotic lesions correlated with the plasma cholesterol level (Fig. 5). However, the extent of atherosclerosis differed dramatically between the groups. The transgenic mice expressing the RK3359-3369SA LDL had strikingly less atherosclerosis than mice expressing the wild-type recombinant LDL. It should be emphasized that the only difference between these two groups of transgenic mice is the mutation of the apo-B gene in the one group that prevents the binding of LDL to proteoglycans and to the LDL receptor. Representative aortas from a single wild-type recombinant LDL mouse, an RK3359-3369SA LDL mouse, and a non-transgenic mouse are shown in Fig. 6. The non-transgenic mouse had essentially no atherosclerosis, a finding that was consistent with the analysis of nine other non-transgenic mice that had been on a high-fat, high-cholesterol diet for 17 weeks.

Example 10 Proteoglycan-LDL Binding Drug Screening Assay

A procedure for a competitive solid-phase plate assay is employed (Edwards, I. et al. 1993. *J. Lipid Res.* 34:1155-1163; and Steele, R. et al. 1987. *Atherosclerosis*. 65:51-62). Normal human plasma LDL (1.0 µg in 50µl of phosphate-buffered saline (PBS) containing 0.01% EDTA per well) is immobilized by absorption to polystyrene 96-well micrometer plates for 18 hours at 4 °C. Excess LDL is removed by washing

in PBS, and nonspecific sites on the plastic are blocked by incubation with PBS containing 5% bovine serum albumin (BSA) for 2 hours at 24 °C. The wells are washed three times with PBS and then with binding buffer (10 mM Tris, 50 mM NaCl, 5 mM CaCl₂, 0.05% BSA). Biotinylated proteoglycans along with a candidate compound are added to each well and incubated for 1 hour at 24 °C. The unbound proteoglycans are removed and the wells are washed three times with 50 mM Tris, 90 mM NaCl, 5 mM CaCl₂, 0.05% BSA before 50µl of streptavidin peroxidase (10µg/ml) is added and incubated for 2 hours at 24 °C. The unbound streptavidin peroxidase is removed and the wells are washed three times with 50 mM Tris, 90 mM NaCl, 5 mM CaCl₂, 0.05% BSA. Finally the peroxidase substrate, chromogen o-dianisidine, is added and absorbency at 405 nm is measured.

Negative control values are obtained by using normal human plasma LDL, or in its place recombinant LDL comprising wild-type human apo-B100, obtained as described above in Examples 1-4. When the proteoglycans are added no candidate compound is added to the negative control wells. Negative control values represent normal LDL proteoglycan binding.

Positive control wells are obtained using the RK3359-3369SA LDL and the K3363E LDL obtained as described above in Examples 1-4 in place of the normal human plasma LDL. Again, when the proteoglycans are added no candidate compound added to the positive control wells. Positive control values represent defective LDL proteoglycan binding.

Those candidate compounds which reduce LDL-proteoglycan binding are identified for further testing and possible use as lead compounds for pharmaceutical development and use.

Example 11

Drug Screening Receptor Binding Assay.

Those candidate compounds which demonstrate disruption of LDL-proteoglycan binding in Example 10 or other LDL-proteoglycan binding assays are tested to ensure that they do not disrupt LDL receptor binding.

Human fibroblasts are cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Seven days before each experiment, the fibroblasts are plated into 12-well cell culture dishes (22-mm diameter per well) at ~12000 cells/well in the same medium. Two days before each experiment, the cells are transferred to DMEM containing 10% human lipoprotein-deficient serum. Normal human ¹²⁵I-labeled LDL (2 µg/ml) along with a candidate compound which has been shown to disrupt LDL proteoglycan binding is added to each well in DMEM containing 25 mM HEPES and 10% human lipoprotein-deficient serum. After a 3-h incubation at 4° C, the medium is removed, and washed three times with DMEM containing 25 mM HEPES and 10% human lipoprotein-deficient serum. The surface-bound radioactivity is determined for each well.

Example 12

Use of apo-B transgenic Mice as

In Vivo Atherosclerosis Model System for

Determining the Efficacy of Candidate Compounds

The transgenic mice described above in Examples 1-3 are used to test the efficacy of candidate compounds at reducing atherosclerosis. Transgenic mice expressing the wild-type human apo-B100 are each administered a candidate compound for 17 weeks, and fed a Paigen diet containing 1.2% cholesterol, 0.5% bile salts, and 20% fat during this period. The mice are then sacrificed, and the aortas are perfusion fixed and analyzed with the *en face* procedure, in which the entire aorta is

pinned out flat, stained with Sudan IV, and analyzed with a morphometric image-analysis system (Image-1/AT) to quantitate the extent of atherosclerosis.

Negative control values are obtained using transgenic mice with the wild-type human apo-B100 which are not administered a candidate compound but are fed the Paigen diet. Positive control values are obtained using transgenic mice expressing the RK3359-3369SA apo-B 100 and the K3363E LDL apo-B100, which can be obtained as described in Examples 1-3 above, which are fed the Paigen diet for 17 weeks.

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